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# POSSIBLE MECHANISM FOR DENERVATION EFFECT ON WOUND HEALING

Annual Report

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We have continued basic research on the role of transferrin, an iron-transport protein required for cell proliferation, in the neural effect on wound healing and tissue regeneration. The system of tissue repair under investigation is the regenerating limb of the axolotl, in which growth is strictly dependent on unknown factors from peripheral nerves. The rationale of the study is to quantify transferrin in segments of ligated normal and regenerating nerves in order to obtain information with which to test the hypothesis that axons transport transferrin to cells of the regenerating tissues.

Before experiments of this nature can be undertaken, axolotl transferrin and antibodies against this factor had to be produced so that immunoassays could be developed to measure this protein in nerves, regenerating limbs, and other tissues from axolotls. These goals were accomplished in the earlier phase of the project and during the past year the immunoassay was used to obtain data regarding axonal transport of transferrin. Results indicate that this protein is part of fast anterograde component of axonal transport in (over)

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the Guide for the Care and Use of Laboratory Animals, prepared by the Committee on Care and Use Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 85-23, Revised 1985).

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## Body of Report

### Statement of the Problem under Study

The program of research supported by this contract the effect of peripheral nerves on cell maintenance, proliferation and growth in developing and adult tissues. Although this influence has been recognized for many years, its biochemical basis is very poorly understood. The major medical importance of this area of investigation lies in the frequent failure of wounds to heal at normal rates in tissues subjected to severe reduction in the nerve supply (1,2).

The animal model being used to study this problem is the urodele amphibian, which has a particularly well-developed capacity for tissue and organ regeneration (2). Appendages of this animal are capable of complete regeneration in a process that is dependent on a neural influence for cell proliferation and growth (2,3). This developmental system is well-suited for investigations of the growth-promoting properties of nerves because the cellular events involved have been carefully characterized histologically (2) and because partial or complete denervation of the regenerating tissue is relatively simple to achieve technically, unlike various commonly used mammalian models of wound healing (3,4).

We are examining the possibility that neural transferrin plays a role in the growth-promoting role of neurons. This factor, a glycoprotein used for iron-transport, is provided to cells throughout the body via plasma and interstitial fluid, and

is required for cell proliferation. The importance of transferrin for proliferation of animal cells is probably due to the iron cofactor requirement of enzymes controlling DNA synthesis (5,6). The observation that peripheral nerves are rich in transferrin (6,7), together with its clear importance for cell survival and proliferation, suggest that an analysis of the availability and possible neural transport of this factor during vertebrate limb regeneration may provide useful new information on the role of nerves in this system.

## Background and Review of the Literature

Early studies, reviewed by Singer (3), indicated several basic features of the neural influence on limb regeneration: it can involve sensory, motor, or autonomic nerves, as well as central nervous tissue; it is needed for cell proliferation and growth of the limb, but not for morphogenesis of the regenerating bud, called the blastema; it is mediated by release of protein factor(s) that are not species-specific. Recent attempts to identify the factor(s) in nervous tissue have used blastemas in organ culture to assay for factors in brain extracts capable of stimulating cell proliferation (8-11), but no protein has yet been purified from nervous tissue using this assay.

A somewhat similar *in vitro* assay has been used to investigate neural factors controlling growth and differentiation of embryonic myoblasts during muscle development (7, 12). Like

limb regeneration, development of skeletal muscle is dependent on unknown protein factors released from nerve (13). The addition of extracts of chick brain or sciatic nerve to culture medium, like co-culture with explanted neural tissue, promote proliferation of cultured myoblasts and fusion of these cells into large, multinucleated myotubes which differentiate into muscle fibers (7). Using this bioassay, Oh and Markelonis (7) purified a protein from sciatic nerve that was capable of promoting the entire sequence of muscle formation and differentiation in vitro. Upon characterization of this factor, which they designated "sciatin", it was found to be the iron-transport factor transferrin (7). Subsequent work showed transferrin to be abundant in peripheral nerves of birds (14) and mice (15). There is evidence that cultured neurons may both take up transferrin by a receptor-mediated mechanism (16) and synthesize the protein (17). It is not yet known whether neural transferrin is critical for myogenesis in vivo and the physiological and developmental significance of transferrin in peripheral nerve remains to be established (6).

By means of the cultured regeneration blastema bioassay, we showed that human serum transferrin is capable of stimulating the mitotic index, the DNA labeling index, and $^{3}H$-thymidine incorporation (18). No effect was found with heat-denatured transferrin or with other serum proteins, such as albumin or immunoglobulin. The addition of transferrin at a concentration of 25 ug/ml to medium containing 1% fetal bovine serum caused all

three parameters to increase to levels normally seen in medium containing 10% serum. The rate of blastema cell proliferation in medium with 10% serum is similar to that *in vivo* and results in extensive outgrowth of cells from the explant. Concentrations of transferrin above the optimal dose were found to inhibit cell proliferation, an effect also observed in dose-response studies with mammalian cells (19).

It has been suggested that the inhibitory effect observed *in vitro* at high transferrin concentrations is due to competition for receptors between iron-free transferrin and iron-carrying transferrin, resulting in reduced delivery of iron to the cells (20). In support of this hypothesis, we found that adding $FeCl_3$ to the medium relieved the inhibitory effect of the high transferrin concentration (18).

Using antiserum to *Pleurodeles* serum transferrin that cross-reacted with newt (*Notophthalmus viridescens*) transferrin, we demonstrated the presence of this protein in extracts of newt brain and peripheral nerve by immunodiffusion (21). Immunohistochemistry of newt ganglia and spinal cord indicated the presence of transferrin in neuronal perikarya and axons (Tomusk and Mescher, unpublished observations).

That neural transferrin may stimulate the cell proliferation seen with brain extracts in the cultured blastema assay is indicated by the finding that removal of the iron from such an extract with the iron chelator desferrioxamine rendered the extract inactive, with full activity restored by the

readdition of ferric iron (21). Moreover, the dose-response curve for brain extract was similar to that of transferrin and the inhibition of growth at high extract concentrations was reversed by $FeCl_3$ (21). These results suggest that transferrin is involved in the stimulatory effect of neural extracts on blastemal cell growth and prompted the present studies on the availability and delivery of transferrin in peripheral nerves during the proliferative phase of normal amphibian limb regeneration *in vivo*.

## Rationale of the Study

The major technical objectives of this project are to purify transferrin from the urodele amphibian, the axolotl (*Ambystoma mexicanum*), to generate antibodies against this protein, and to develop an enzyme immunoassay for determining the concentration of this protein in axolotl tissue extracts. The rationale for this plan is that if significant release of transferrin from nerves occurs in the limb, then denervation of the limb should lower the transferrin concentration in limb tissues. Similarly, measurement of transferrin concentrations at different regions in ligated sciatic nerves should provide evidence regarding the possible axoplasmic transport of this factor.

Moreover, the availability of antiserum against axolotl transferrin is expected to allow better immunohistochemical

localization of transferrin in axolotl nerves and regenerating limb tissues than was possible with the antiserum against Pleurodeles transferrin used in our earlier studies.

Experimental Methods

The content of transferrin in a peripheral nerve of the axolotl limb was determined using a noncompetitive, sandwich-type enzyme-linked immunosorbent assay (ELISA) developed in the previous phase of this project. A brief description of the method used for the ELISA is as follows. Wells of a 96-well microtiter plate (Falcon Plastics) were coated overnight at 4°C with rabbit antiserum against purified axolotl transferrin at a dilution of 1:1500. The plate was then washed three times with Tris-buffered saline containing 0.05% Tween (TBS/Tween). Wells were then blocked with 5% bovine serum albumin (Sigma Chemical) for 1 hour and washed again three times with TBS/Tween. Samples of supernatants from axolotl nerve homogenates were then added to the wells in triplicate along with standard concentrations of purified axolotl transferrin, in duplicate. Plates with standards and tissue samples were incubated at 4°C overnight, after which they were washed again in TBS/Tween. The mouse antiserum against axolotl transferrin was added at a dilution of 1:2000 and incubated at room temperature for 2-3 hours. Plates were then washed again three times in TBS/Tween. A 1:500 dilution of secondary antibody (goat anti-mouse IgG) conjugated to alkaline phosphatase (Sigma Chemical) was added to the wells and incubated

1 hour. Plates were washed three times with TBS/Tween. An alkaline phosphatase substrate, para-nitrophenyl phosphate (Sigma Chemical), was added to each well at 1 mg/ml and incubated 30 min at room temperature. The colorigenic reaction was stopped by adding 3M NaOH after 30 min and the absorbance in the wells was measured at 405 nm and 540 nm with a dual wavelength spectrophotometric plate reader (Model EL309, Bio-Tek Instruments). Protein content of the tissue extracts was also determined by the method of Smith et al. (22).

To provide regenerating nerve in sufficient amounts for use in the ELISA, hindlimbs of adult axolotls (Ambystoma mexicanum), anaesthetized by immersion in 0.1% benzocaine (Sigma Chemical), were amputated unilaterally just proximal to the ankle and allowed to regenerate for various periods of time. The contralateral hindlimb was left intact as a control. Animals were maintained at 20°C on a 12 hour light/12 hour dark cycle. Limbs were used at three different periods of regeneration (early bud, palette, and digital outgrowth), which are stages in the system described by Tank et al. (23). These stages of regeneration were reached at approximately eight, ten, and twelve weeks after amputation respectively.

At the times indicated, animals were re-anaesthetized and each sciatic nerve was exposed above and below the level of the knee. Using 4-0 silk suture material, two ligatures 9 mm apart were placed on the nerve at this level, with the distal ligature at least 3 mm from the level of amputation (Fig. 1). After

reclosing the skin, the animals were maintained in oxygenated water at room temperature (22°C) for three hours.

At this time the animal was anaesthetized again and both sciatic nerves were removed, cleaned of investing epineurium, and cut into 3 mm segments between and on each side of the ligatures. (Animals were then sacrificed by decapitation.) Individual segments of sciatic nerve were then homogenized, using the buffer system of Meek and Adamson (15). The transferrin content of each homogenate was determined in the ELISA described above.

As a control that any transferrin accumulation obtained at the ligatures was due to axonal transport rather than to edema or vascular blockage, colchicine was prepared in a slow-release solid matrix of ethylene vinyl acetate (a gift of Dr. R. Laufer, MIT) and implanted at a level just distal to the lumbar plexus. Colchicine disrupts microtubules and thus inhibits accumulation of axonally transported material at neural ligatures.

Employing methods developed by others for quantification of axonal transport rates in mammalian peripheral nerves (24, 25), transferrin concentrations in the segments from each nerve were used to determine the percentage of neural transferrin that was involved in axonal transport (the mobile fraction) and the rate at which this factor was transported in both the retrograde and anterograde directions.

## Results

The mean concentration of transferrin in the segments of

adult axolotl sciatic nerves was found to increase dramatically during the process of nerve and limb regeneration (Table I). Unilateral amputation caused the transferrin concentration in the affected sciatic nerve to increase approximately 3-fold by the early bud stage of limb regeneration, 7-fold by the palette stage, and 20-fold by the digital outgrowth stage. Somewhat smaller increases in transferrin content also occurred in the contralateral intact sciatic nerves during this period, suggesting stimulation of transferrin uptake or synthesis in these intact nerves by an unknown humoral factor produced in response to the contralateral limb or neural injury.

Transferrin concentrations in nerve segments from regenerating limbs were analyzed in relation to the ligatures. Taking the average concentration between the ligatures as 100%, the two segments proximal to the first ligature were found to contain approximately 250% of this amount (Fig. 2). This result implies that transferrin was being transported in the anterograde (proximal to distal) direction during the three hour period of ligation. No accumulation was found distal to the second ligature, suggesting little or no retrograde transport of this protein. The actual mean concentrations of transferrin in the segments proximal and distal to the ligatures from nerves at the three stages of limb regeneration examined are given in Table II.

To investigate the basis for the accumulation of transferrin at the ligature, colchicine was applied to the sciatic nerve at a level distal to the lumbar plexus using a

polymer designed for the slow release of incorporated compounds. Colchicine was prepared in the polymer (Elwax) at a concentration of 10 mg/cc and a 0.5 cc implanted was used for each limb. As shown in Figure 3, this treatment 3 days before ligation completely inhibited accumulation of transferrin at the ligature during the three hour period of nerve ligation. This indicates that the accumulation is due to microtubule-based axonal transport, rather that vascular blockage or edema produced by the ligature. This control experiment was particularly important in this axonal transport study because the protein under investigation is an abundant plasma protein and therefore might be expected to become concentrated at sites of edema or blood accumulation.

The average transferrin concentrations proximal, distal, and between the ligatures were used to calculate the mobile fraction and transport rate of this protein in both regenerating and intact nerves. Although this concentration increased in the nerves during limb regeneration, the percentage of transferrin undergoing axonal transport in the nerves (the mobile fraction) did not change significantly during this period (Table II). The mobile fraction of this protein in control intact nerves was also not significantly lower than that of regenerating nerves (Table II).

The rate of transferrin transport in the anterograde direction was also similar in the regenerating nerves at all three stages of limb regeneration and in all intact nerves

(except those in the early bud stage group). This rate averaged 70-75 mm/day or 3 mm/hour, which clearly indicates that transferrin is carried in the fast component of axoplasmic transport which involves proteins carried in vesicles.

Discussion

This study represents the first quantitative examination of transferrin in injured, regenerating peripheral nerves. It is also the first axonal transport study of any plasma protein. The observation that the concentration of this protein increases greatly in nerves during their regeneration is relevant to the requirements for rapid metabolism at growth cones of regenerating axons. The large number of mitochondria in growth cones suggests a requirement for iron which is used as the cofactor in several mitochondrial respiratory enzymes. In support of this idea, a correlation has recently been shown between the accumulation of transferrin and the formation of new mitochondria in developing neurons in rats (26). The need for new mitochondria during axonal growth may dictate increased uptake of the iron-transport factor. In adult rat peripheral nerve, the onset of regeneration induced by crush injury has recently been shown to stimulate a rapid increase in the number and density of transferrin receptors on the neuronal cell bodies and this increase is followed by an accumulation of iron by the regenerating neurons (27). Our results showing increased transferrin accumulation in regenerating axolotl sciatic nerves are consistent with this report. The increased number of transferrin receptors suggests

that the concentration of transferrin in nerves is due to increased uptake by the neurons, rather than to local synthesis of the protein by neurons or Schwann cells.

The increased amounts of transferrin in regenerating nerves which we have demonstrated provides a reasonable explanation for the greater growth-promoting activity of such nerves compared to intact nerves, which has been reported by others. Early studies by Singer (4) showed that the motor nerve component of salamander forelimbs alone was not capable of supporting limb regeneration, but that if the motor nerves themselves were regenerating as a result of a previous injury they did have the capacity by themselves to support blastema formation and limb regeneration. This result was confirmed more recently and extended by Maier et al. (28). Also, in a study of quite different experimental design, it has been shown that extracts of regenerating neural tissue have greater mitogenic activity for cultured blastema cells than similar extracts of normal neural tissue (29). The basis of this increased growth-promoting activity in nerves during their own regeneration remains unclear, but the present results with transferrin suggest for the first time a mechanism that is not only plausible physiologically, but strongly supported by what is known regarding requirements for axonal growth and cell proliferation.

Why the transferrin concentration in the contralateral intact nerves was also affected by amputation is not clear. However, similar "transneuronal" effects have been reported in

several other studies of neuronal response to unilateral injury. Using another species of salamander Tweedle (30) examined the effect of unilateral forelimb amputation on neuronal cell bodies of both motor and sensory nerves. Both injured and intact nerves showed similar chromatolytic reactions and increased RNA synthesis. The bilateral effect on nerves following unilateral injury suggests that the response to injury involves release of a humoral factor that stimulates a multifaceted neuronal activation.

Our studies on axonal transport of transferrin represent the first work examining such transport of any protein during limb regeneration in the amphibian. Previous work, reviewed by Wallace (31), suggested that the trophic factor promoting blastema growth is in fact transported in the fast component of the axons, but this possibility has not been tested prior to this study. The data obtained from the double ligature study indicate that transferrin is transported along axons in a proximal-to-distal direction and that retrograde transport of this factor, if it occurs at all, is much less important quantitatively. In two previous preliminary single ligation studies of axonal transport, by Oh and Markelonis (7) using chickens and by Mescher and Tomusk (unpublished) using adult rats, transferrin transport was observed in both directions. The cause of these disparities in results, possibly owing to differences in methodology or species, and its significance remain to be resolved. Whatever the cause, the apparent lack of retrograde transport in the axolotl does not

affect the utility of the present results in testing the hypothesis that is the basis for this project.

Since transferrin is a protein very abundant in blood and lymph, the control experiment involving the effect of colchicine on accumulation at the ligatures was particularly important. It is possible that ligating a sciatic nerve could produce vascular blockage or edema at the ligation site, which would likely produce increased local concentrations of all plasma proteins. Accumulation due to this, however, would still occur in the presence of the microtubule inhibitor colchicine. The observation that colchicine blocked accumulation at the ligature strongly suggests that such accumulation is due to transport of transferrin along microtubules in axons, as in the fast component of axonal transport. This conclusion is further strengthened by the observation that accumulation occurred on one side of the ligature only; protein accumulation due to edema or vascular constriction typically occurs in both directions (32).

The distal transport of this growth-promoting factor is significant for understanding the permissive effects axons exert on proliferating blastema cells. If the quantity of axonally transported Tf that was found to accumulate at the proximal ligature was released within an early bud blastema at that level, a local concentration would be maintained within the blastema that correlates very well with the optimal Tf concentration for proliferation in vitro (19,27).

The rate of transferrin transport, as calculated from the

ligation data, clearly puts this protein in the fast component and is similar to the rate reported in other axonal transport studies using amphibian nerves (33, 34) As discussed above, such a transport rate for the growth-stimulating factor of nerves has been predicted from earlier work on the neural effect on blastema formation (31). This rate is also consistent with the result found earlier in this project that brachial nerve axotomy reduces the transferrin concentration in forelimb blastemas within one day of the operation. Moreover, the rate of transport was similar in both intact and regenerating, despite the higher concentration of transferrin during regeneration, and this observation is also consistent with other studies of axonal transport in regenerating nerves (35).

Summary and Conclusions

The primary reagents generated in the first phase of this project, the antibodies against axolotl transferrin, were employed during the second phase to develop a rapid and sensitive ELISA for measurement of transferrin in tissue extracts and to localize this protein in axons and Schwann cells by light microscopy. During the last phase of the project the ELISA has been used in conjunction with double ligation studies on axolotl sciatic nerves to determine the direction, magnitude, and rate of axonal transport of transferrin in the major nerve supply to the regenerating limb. This work represents the first study of axonal transport of a protein important for growth in regenerating

amphibian limbs.

The results show clearly that neural transferrin is transported in axoplasm, in the anterograde direction, and at a rate expected of the mechanism involving proteins in vesicles transported along microtubules (fast component). Such transport of the trophic material for limb regeneration has long been hypothesized.

These results strongly support the hypothesis concerning a role for neural transferrin in limb regeneration. If transferrin undergoes exocytosis from axons, as it does from other cells after release of iron to cytoplasmic factors, the released protein will bind additional ferric ion and be available to meet the iron requirements of local cells. If transferrin is not available in sufficient concentration from the plasma, then a dependence on axons for this factor supporting growth may be apparent. The early, nerve-dependent limb regeneration blastema is avascular (2). This, together with the proteolytic nature of the blastema's extracellular environment (2), suggests that the supply of plasma proteins such as transferrin from capillaries may be reduced locally. Transferrin released from axonal growth cones, which are abundant among the cells of the blastema (2), would supplement that available from plasma. The axonally secreted protein would bind iron released from local sources, such as degenerating erythrocytes or other cells, and then bind receptors on blastema cells to deliver the iron needed for their for proliferation.

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Table I

**TRANSFERRIN CONCENTRATIONS (ng/mm) IN SCIATIC NERVES FROM REGENERATING AND CONTRALATERAL CONTROL LIMBS**

| Stage of Regeneration | Regenerating | Control |
|---|---|---|
| (unamputated) | ---- | 9.1 ± 1.1 (3) |
| early bud | 28.1 ± 4.0 (4) | 14.3 ± 2.4 (4) |
| palette | 62.7 ± 11.4 (4) | 54.4 ± 13.9 (4) |
| digital outgrowth | 213.7 ± 49.4 (3) | 159.3 ± 19.1 (3) |

**Table II**

**AXONAL TRANSPORT OF TRANSFERRIN AT THREE STAGES OF LIMB REGENERATION[1]**

| Transferrin in sciatic nerve | EARLY BUD | PALETTE | DIGITAL OUTGROWTH |
|---|---|---|---|
| Average concentration (ng/mm) | 28.1 ± 4.0 (4)[2] | 62.7 ± 11.4 (4) | 213.7 ± 49.4 (3) |
| Concentration proximal to ligature (ng/mm) | 54.3 ± 16.9 (4) | 126.8 ± 47.7 (4) | 354.4 ± 73.2 (3) |
| Concentration distal to ligature (ng/mm) | 10.2 ± 3.6 (4) | 26.1 ± 13.1 (4) | ---- |
| Mobile fraction | 0.41 ± 0.15 (4) | 0.39 ± 0.18 (3) | 0.22 ± 0.10 (3) |
| Anterograde transport rate (mm/d) | 74.7 ± 33.9 (4) | 75.8 ± 43.8 (3) | 76.9 ± 27.5 (3) |

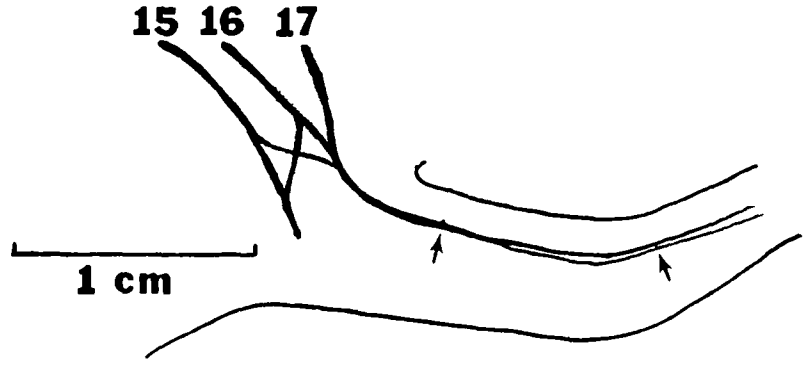


Figure 1. Schematic of axolotl sciatic nerve emerging from spinal nerves 15, 16, and 17 with arrows indicating positions of ligations for axonal transport studies.

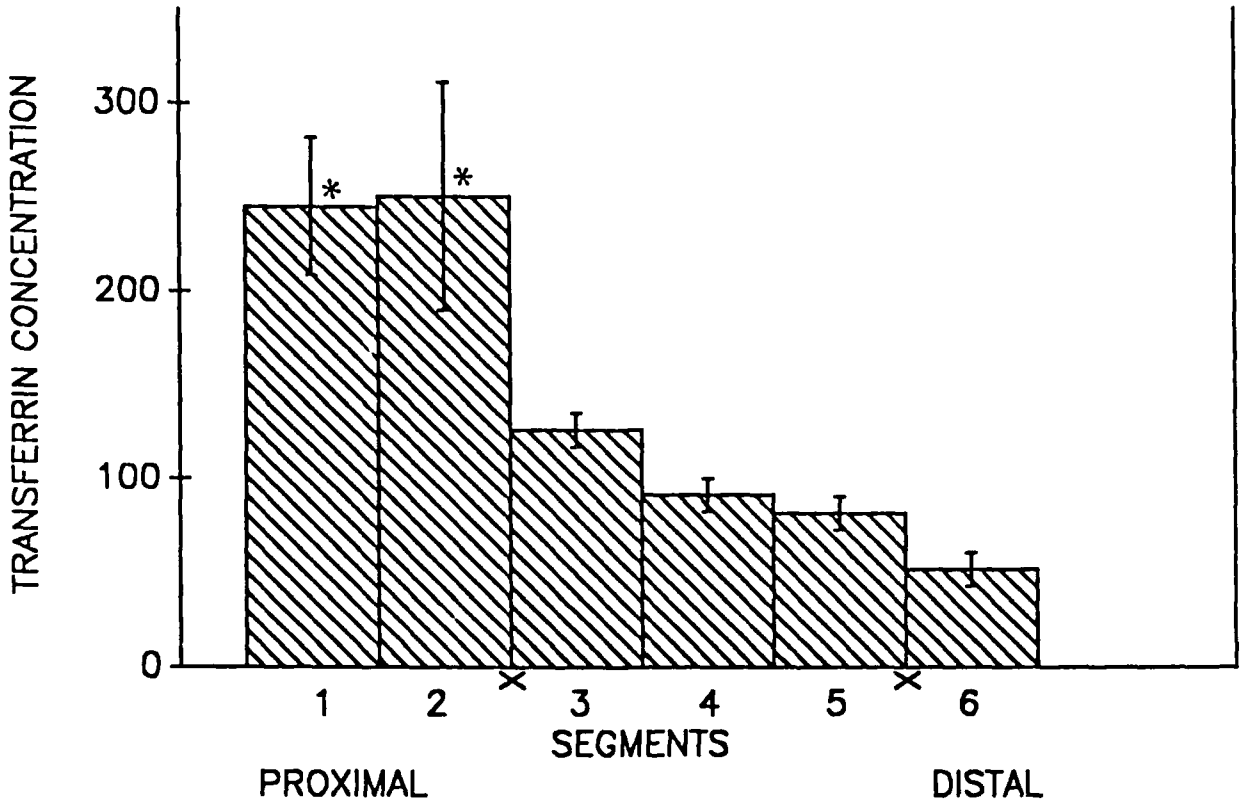


Figure 2. Transferrin concentrations in segments of regenerating sciatic nerves 3 hours after ligation, as percentages of the average concentration of the 3 segments between the ligatures (x's). Each bar indicates mean ± s.e. of 11 segments.

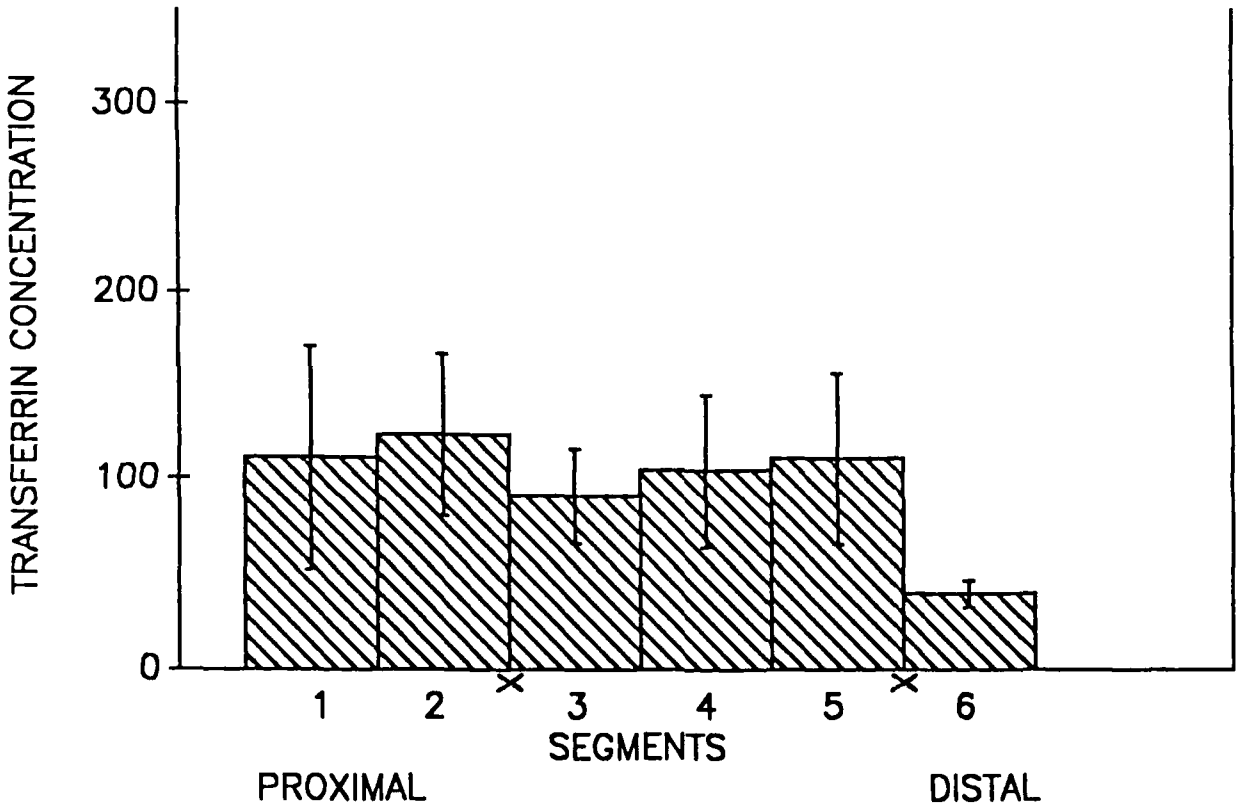


Figure 3. Transferrin concentrations of nerve segments as in Figure 2 but following local treatment of nerves with colchicine distal to lumbar plexus. Each bar indicates mean ± s.e. of 4 segments.

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